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Title Page

Arresting the Development of Addiction: The Role of β-Arrestin2 in Drug Abuse

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Running Title Page

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List of abbreviations:
βarr – beta-arrestin
βarr1 – beta-arrestin1
βarr2 – beta-arrestin2
βarr2-KO – beta-arrestin2 knock-out
CPP – conditioned place preference
Dbh -/- – dopamine β-hydoxylase knock-out
DREADD – Designer Receptors Exclusively Activated by Designer Drugs
GPCRs – G protein-coupled receptors
LSD – lysergic acid diethylamide
MSNs – medium spiny neurons
PCP – phencyclidine
Serotonin – 5-HT
SNP – single nucleotide polymorphism

Recommended Section Assignment: Minireview

Abstract

The protein β-arrestin2 (βarr2) directly interacts with receptors and signaling pathways that mediate the behavioral effects of drugs of abuse, making it a prime candidate for therapeutic interventions. βarr2 drives desensitization and internalization of G protein-coupled receptors, including dopamine, opioid, and cannabinoid receptors, and can also trigger G protein-independent intracellular signaling. βarr2 mediates several drug-induced behaviors, but the relationship is complex and dependent on the type of behavior (e.g., psychomotor vs. reward), the class of drug (e.g., psychostimulant vs. opioid), and the circuit being interrogated (e.g., brain region, cell type, and specific receptor ligand). Here we discuss the current state of research concerning the contribution of βarr2 to the psychomotor and rewarding effects of addictive drugs. Next we identify key knowledge gaps and suggest new tools and approaches needed to further elucidate the neuroanatomical substrates and neurobiological mechanisms to explain how βarr2 modulates behavioral responses to drugs of abuse, as well as its potential as a therapeutic target.

Introduction

G protein-coupled receptors (GPCRs) mediate many of the neurochemical and behavioral effects of addictive drugs. For example, most drugs of abuse increase dopamine neurotransmission in the mammalian brain, either directly or indirectly, which leads to the stimulation of dopaminergic GPCRs (Di Chiara & Imperato, 1988; Luscher & Ungless, 2006; Pierce & Kumaresan, 2006). Some classes of drugs, such as opioids and cannabinoids, are agonists at opioid and cannabinoid receptors, respectively, which are also GPCRs. GPCR signaling is thus a critical component of drug-induced neurotransmission.

Termination of signal transduction at GPCRs is necessary to prevent continual signaling and to allow receptors to be reactivated by ligands. Arrestins, which were first discovered in photoreceptor-expressing cells in the eye (Dolph, 2002), are proteins that bind to active phosphorylated GPCRs and inactivate them (Gurevich & Gurevich, 2004). Visual arrestins are located primarily in the eye, and non-visual arrestins, also known as β-arrestins (βarrs), are ubiquitously expressed. βarrs, so named from the discovery of their interactions with β-adrenergic receptors (Attramadal *et al.*, 1992), are scaffolding proteins involved in the desensitization and internalization of GPCRs at the plasma membrane. They can also initiate intracellular signaling cascades independent of canonical G protein signaling (Smith & Rajagopal, 2016). GPCRs in the membrane were thought to signal until βarr desensitized and internalized them; however, recent experiments have shown that certain GPCRs can signal after being internalized by βarr into endosomes (Irannejad *et al.*, 2013), and βarr can even potentiate Gs signaling (Wehbi *et al.*, 2013). βarrs can interact with ERK, Atk, MEK, Raf-1, JNK, and ubiquitin ligases, to name a few (Luttrell *et al.*, 2001; Shenoy *et al.*, 2001; Beaulieu *et al.*, 2005; Del'Guidice *et al.*, 2011; Urs *et al.*, 2011; Kuhar *et al.*, 2015). βarr can also modulate NF-κB

through IκBα (Gao *et al.*, 2004). Additional interactions and complexities of βarr continue to be discovered; therefore, the field's understanding of βarr is constantly evolving.

Functional selectivity, also known as biased agonism, is the principle that agonists bound to a GPCR can preferentially signal through different pathways. GPCR ligands can selectively activate intracellular G protein signaling, βarr pathways, or both to varying degrees (Luttrell *et al.*, 2015). Biased agonism complicates GPCR activity, as different agonists at the same receptor can have opposing effects on physiology (Boerrigter *et al.*, 2012; Tarigopula *et al.*, 2015). Ligands that preferentially engage βarr versus G protein signaling can alter responses to opioids, psychostimulants, and hallucinogens (Soergel *et al.*, 2014; Peterson *et al.*, 2015; Manglik *et al.*, 2016; Urs *et al.*, 2016); therefore, biased agonists could be potential therapeutics for addiction, analgesia, and other conditions.

There are two βarrs: β-arrestin1 (βarr1; also known as arrestin-2) and β-arrestin2 (βarr2, also known as arrestin-3). βarr1 and βarr2 can have similar or distinct roles in cells, and their function can vary by cell type. For example, reduction of either βarr1 or βarr2 decreased β2-adrenergic induced ERK signaling (Shenoy *et al.*, 2006). However, angiotensin II receptor mediated ERK signaling decreased with reduction of βarr2 but increased with downregulation of βarr1 (Ahn *et al.*, 2004). Most class A receptors (among them dopamine, opioid, and cannabinoid receptors) bind both βarr1 and βarr2; however, they have a higher affinity for βarr2 (Oakley *et al.*, 2000), making βarr2 a likely candidate for modulating GPCR signaling and the effects of drugs of abuse.

Indeed, mice lacking βarr2 but not βarr1 have altered behavioral responses to addictive drugs, including morphine (Bohn *et al.*, 2003; Urs & Caron, 2014), amphetamine (Urs & Caron,

2014), and alcohol (Li *et al.*, 2013) (Table 1); therefore, βarr2 a likely candidate for modulating the effects of drugs of abuse. The purpose of this review is to catalog what is currently known about the role of βarr2 in mediating the behavioral effects of various drugs of abuse and to discuss the questions that remain and what techniques are needed to properly answer these questions. For a more thorough review of the cellular and molecular functions of βarr2, we refer the reader to (Smith & Rajagopal, 2016).

βarr2 Modulates the Effects of Multiples Classes of Drugs of Abuse

Opioids.

Most of the pioneering studies that examined the role of βarr2 in the behavioral effects of drugs of abuse used conventional βarr2 knockout (βarr2-KO) mice, which completely lack βarr2 in all cells throughout development and in adulthood. βarr2-KO mice have significantly blunted morphine-induced locomotion but enhanced morphine reward, as measured by conditioned place preference (CPP) (Bohn *et al.*, 2003).

Because many psychomotor drug effects are mediated by dopamine neurotransmission in the striatum and prefrontal cortex (Pierce & Kumaresan, 2006), βarr2 is presumed to exert its effects in the mesocorticolimbic system. Indeed, βarr2-KO mice exhibit higher morphine-induced dopamine release in the striatum compared to controls (Bohn *et al.*, 2003). βarr2 in medium spiny neurons (MSNs) that contain dopamine receptors in the dorsal and ventral striatum may also modulate the psychomotor effects of drugs. However, the relative contribution of the D1 or D2 family of dopamine receptors to the differing drug-induced behavioral effects is unknown. Determining which family of dopamine receptors mediates such effects is important because D1 and D2 receptors have opposing influences on the excitability of MSNs (Beaulieu &

Gainetdinov, 2011) and are anatomically segregated onto separate populations of MSNs that project to different brain regions (Lobo & Nestler, 2011). Indeed, activation of D1- versus D2-containing MSNs causes differential changes in whole brain activity (Lee *et al.*, 2016) and has opposite effects on reinforcement (Kravitz *et al.*, 2012).

βarr2 in D1-containing neurons was initially proposed as a mechanism for morphine-induced locomotion. In support of this hypothesis, morphine normally induces a βarr2/pERK signaling complex, but this did not occur in D1-KO mice (Urs *et al.*, 2011). Additionally, D1-KO mice and wild-type animals developed a similar CPP for morphine (Urs *et al.*, 2011), demonstrating that D1 receptors are not necessary for morphine reward. To directly test the role of βarr2 in D1-containing neurons, morphine CPP and locomotion could be tested in conditional KO mice that lack βarr2 only in D1-containing cells (Urs *et al.*, 2016). Similarly, future experiments should directly test the role of βarr2 in D2-containing neurons in mediating the effects of morphine.

While βarr2's interactions with dopamine receptors may modulate opioid effects, μ-opioid receptor recruitment of βarr2 could also be important because morphine has a high affinity for μ-opioid receptors, which, like dopamine receptors, are expressed on striatal MSNs, among other regions. Historically, though, morphine was found to not recruit βarr2 and prompt the internalization of μ-opioid receptors as readily as many other μ-opioid receptor agonists. For example, etorphine caused robust internalization of μ-opioid receptors in the cortex of rats 30 min post-administration, whereas morphine caused no detectable μ-opioid receptor endocytosis (Keith *et al.*, 1998). Similarly, previous in vitro experiments did not find morphine-induced recruitment of βarr2 or the internalization of μ-opioid receptors in HEK cells, unless: GPCR kinase was overexpressed (Zhang *et al.*, 1998), βarr was overexpressed, or βarr1 was eliminated

and only βarr2 was available (Whistler & von Zastrow, 1998). However, morphine did trigger the rapid endocytosis of μ-opioid receptors in striatal neurons (Haberstock-Debic *et al.*, 2005). Despite morphine's reduced ability to recruit βarr2 in some cells, βarr2 is critical for morphine's effects. In vitro experiments using βarr2-KO, βarr1-KO, and control cells revealed that, unlike the selective μ-opioid receptor agonist DAMGO, which can recruit either βarr1 or βarr2 to μ-opioid receptors, morphine appears to be able to recruit only βarr2. Indeed, βarr2 but not βarr1 internalized morphine-activated μ-opioid receptors (Groer *et al.*, 2011). Together, these studies indicate that μ-opioid receptors recruit βarr2 in certain cell populations, such as MSNs, but not in others, and sensitive assays are needed to observe these effects. Furthermore, different opioids can elicit unique adaptations that alter how βarr2 is engaged and highlight the importance of biased agonism in understanding βarr2 recruitment.

The ability or inability of μ-opioid agonists to recruit βarr2 can have important behavioral consequences. The μ-opioid receptor agonists morphine, heroin, and oxycodone have low recruitment of βarr2 and low levels of endocytosis (Keith *et al.*, 1998; Whistler & von Zastrow, 1998; Zhang *et al.*, 1998); however, all three have high abuse potential and can cause tolerance and dependence. It has therefore been hypothesized that the lack of opioid-induced desensitization and internalization of μ-opioid receptors contributes to these features of addiction. To test this hypothesis, a mutant recycling μ-opioid receptor that desensitizes and internalizes in response to morphine was created (Finn & Whistler, 2001). Interestingly, while these mice had an enhanced CPP to low doses of morphine, they neither escalated morphine consumption nor exhibited aberrant motivation for drug over weeks of morphine self-administration, unlike wild-type mice, which escalated intake, continued to self-administer drug despite the threat of footshock, and had greater reinstatement of drug seeking (Berger &

Whistler, 2011). These results demonstrated that μ-opioid receptor internalization has different effects on drug reward (as measured by CPP) versus the development of more complex addictive-like behaviors. Because βarr2 is recruited to morphine-activated receptors to internalize them (Groer *et al.*, 2011), it may play an important role in preventing the transition from recreational use to addiction. Future experiments should investigate this directly.

To determine how endogenous βarr2 changes with morphine exposure, in situ hybridization was conducted on the brains of rats with a history of morphine administration. Chronic but not acute morphine exposure increased βarr2 mRNA in cortex and decreased βarr2 mRNA in the periaqueductal gray (Fan *et al.*, 2003). Additionally, naloxone-precipitated withdrawal robustly increased βarr2 mRNA in hippocampus (Fan *et al.*, 2003). These experiments revealed that βarr2 can be differentially regulated in various brain regions in response to drugs. Future experiments are needed to determine the behavioral and biological effects of changing βarr2 levels in these and other brain regions.

Consistent with the animal research indicating that βarr2 modulates drug effects, human studies have found differences in βarr2 in people susceptible to opioid abuse. Indeed, individuals who died from heroin overdose had decreased levels of βarr2 specifically in the prefrontal cortex compared to matched controls (Ferrer-Alcon *et al.*, 2004). In addition, a haplotype block that spans the βarr2 locus has been discovered, and four single nucleotide polymorphisms (SNPs; rs34230287, rs3786047, rs1045280 and rs2036657) have been studied in opioid-dependent humans under treatment. Those who were homozygous for the variant allele of any three of the four SNPs (all but rs34230287) were more likely to continue using opioids or cocaine while on methadone maintenance treatment (Oneda *et al.*, 2011). This demonstrated that genetic differences in βarr2 can confer resistance to methadone treatment for opioid dependence. None

of these SNPs cause changes in the amino acid sequence, and the biological effects of each are not yet known; however, different SNPs could reduce, enhance, or have no effect on GPCRs and βarr2 signaling. Future research should investigate the physiological effects of each of these SNPs and whether they cause changes in βarr2 expression in different brain regions -.

Chronic pain is a common reason why individuals begin taking opioids, which can transition into opioid addiction; therefore, examining the role of βarr2 in opioid modulation of pain is an important consideration. Paradoxically, μ-opioid receptor agonists that readily recruit βarr2, such as fentanyl, methadone, and etorphine, caused similar analgesia in wild-type and βarr2-KO mice; however, agonists that do not robustly recruit βarr2, such as morphine and heroin, enhanced analgesia in βarr2-KOs compared to controls (Bohn *et al.*, 1999; Bohn *et al.*, 2004a). Similarly, both wild-type and βarr2-KO mice developed tolerance to fentanyl, oxycodone, and methadone (Raehal & Bohn, 2011) but not to morphine following chronic administration (Bohn *et al.*, 2002; Raehal & Bohn, 2011). Although βarr2 is not readily recruited by morphine in all cell types, it can play a critical role in desensitizing μ-opioid receptors (Bohn *et al.*, 2002; Bohn *et al.*, 2004a; Groer *et al.*, 2011).

βarr2 likely mediates many of morphine's negative side effects. In supporting of this idea, βarr2-KO mice have significantly reduced morphine-induced constipation and respiratory suppression, compared to controls (Raehal *et al.*, 2005). Additionally, after screening over 3 million molecules as new potential opioids, the compound PZM21 was found to have high selectivity for the μ-opioid receptor and to strongly activate Gi signaling without engaging βarr2 (Manglik *et al.*, 2016). Interestingly, PZM21 was an effective analgesic in the hotplate test (which engages brain and spinal pain circuits) but not in the tail-flick test (which solely engages spinal, reflexive circuits), suggesting that this novel opioid causes affective but not reflexive

analgesia. Unlike morphine, which has high abuse potential, doses of PZM21 that cause analgesia did not increase locomotor active or support a CPP. Combined, these experiments demonstrate that μ-opioid receptor agonists that preferentially engage Gi, but not βarr2, signaling could be effective treatments for pain without the negative side effects and high abuse potential.

For a more comprehensive review of βarr2's involvement in opioid-mediated analgesia, please refer to (Raehal & Bohn, 2014). Because βarr2 is differentially involved in the antinociceptive effects of various opioids, future experiments should test the role of βarr2 in the rewarding effects of opioids other than morphine. Additionally, functionally selective agonists such as PZM21 could be used in future experiments to preferentially engage Gi signaling following μ-opioid receptor activation.

Cannabinoids

As with morphine, cannabinoid receptor agonists can provide pain relief and are abused. Both CB1 and CB2 cannabinoid receptors recruit βarr2 (McGuinness *et al.*, 2009; van der Lee *et al.*, 2009; Turu & Hunyady, 2010; Delgado-Peraza *et al.*, 2016). Most research has focused on CB1 (Daigle *et al.*, 2008; Mahavadi *et al.*, 2014; Delgado-Peraza *et al.*, 2016), while very little is known about potential contribution of βarr2 to the effects of CB2 activation (Atwood *et al.*, 2012). βarr2 is involved in the behavioral effects of some but not all cannabinoid receptor agonists (Raehal & Bohn, 2014). The cannabinoid receptor agonist Δ^9-tetrahydrocannabinoid (THC) caused greater antinociception in βarr2-KO mice compared to controls (Breivogel *et al.*, 2008; Nguyen *et al.*, 2012), but no differences between genotypes were observed for the CB1 agonists CP55940, methanandamide, JWH-073, and O-1812 (Breivogel *et al.*, 2008). Seven-day

treatment with CP55940 increased βarr2 mRNA and protein levels in the prefrontal cortex (Franklin *et al.*, 2013), suggesting that cannabinoid agonists can upregulate βarr2.

To our knowledge, the role of βarr2 in the rewarding effects of cannabinoids has yet to be examined. Due to the growing use and legalization of marijuana, future experiments should examine the role of βarr2 in modulating cannabinoid reinforcement and use.

Psychostimulants

The first published experiments examining the role of βarr2 in behavioral responses to psychostimulants were conducted using βarr2-KO mice. No robust differences in cocaine-induced locomotion, locomotor sensitization to cocaine, or cocaine CPP were documented in βarr2-KO mice compared to wild-type controls (Bohn *et al.*, 2003). βarr2-KO mice appeared to have slightly reduced cocaine-induced locomotion, but this is difficult to interpret given baseline differences in locomotor activity between genotypes (Bohn *et al.*, 2003; Bohn *et al.*, 2004b). However, βarr2-KO mice had significantly blunted amphetamine-induced locomotion, and this effect was not due to differences in stereotypy (Beaulieu *et al.*, 2005). Similarly, mice lacking βarr2 in all neurons (generated by crossing floxed βarr2 mice with CMV-Cre mice) showed impaired amphetamine-induced locomotion compared to controls (Urs *et al.*, 2016). There are a few possible explanations as to why differences in locomotion were observed following amphetamine but not cocaine in mice lacking βarr2. If βarr2 deletion had a real but more subtle effect on cocaine-induced locomotion than amphetamine-induced locomotion, collapsing the data and analyzing 90-min time bins could obscure the effect. Additionally, it is possible that βarr1 compensates in the absence of βarr2 to facilitate cocaine- but not amphetamine-induced locomotion. Given the different ways that cocaine and amphetamine interact with the dopamine

transporter (reuptake blocker versus substrate/releaser, respectively) and the regulation of dopamine transporter function by intracellular signaling (Schmitt & Reith, 2010), it is plausible that βarr2 interacts either directly or indirectly with the dopamine transporter in such a way that cocaine and amphetamine are differentially affected, although we are not aware of data to substantiate this explanation.

As detailed in the opioid section, determining whether βarr2 influences drug effects via D1- or D2-containing neurons is important due to these receptors' opposing effects and projections to different brain regions. Eliminating βarr2 only in certain populations of neurons is possible by crossing floxed βarr2 mice with D1Cre mice (for D1-containing cells), D2Cre mice (for all D2-containing cells), A2aCre mice (for D2-containing post-synaptic striatal neurons), or ChaTCre mice (for cholinergic interneurons). Mice lacking βarr2 in striatal D2 MSNs or in all D2-containing neurons but not in D1-containing neurons or cholinergic interneurons exhibited blunted amphetamine-induced locomotion (Urs *et al.*, 2016). These experiments demonstrated that βarr2 in D2-containing neurons, particularly in the striatum, facilitates amphetamine locomotor responses. To further probe the role of βarr2 in behavioral responses to amphetamines, viral vectors have been created to express D2 receptors with biased G protein signaling or D2 receptors with biased βArr2 signaling. Overexpressing D2 receptors with biased βarr2 but not G protein signaling in the striatum potentiated amphetamine-induced locomotion (Peterson *et al.*, 2015), revealing a role of βarr2 signaling downstream of striatal D2 receptors. Together, these experiments demonstrated that βarr2 modulates the locomotor-activating effects of psychostimulants, likely through βarr2 signaling in D2-containing cells. Future experiments should test whether this mechanism also mediates psychostimulant reward.

Mice that lack dopamine β-hydroxylase (*Dbh -/-* mice) cannot synthesize norepinephrine and are hypersensitive to cocaine. Interestingly, these animals had reduced βarr2 in the nucleus accumbens and altered D2 receptor function (Gaval-Cruz *et al.*, 2014). Overexpressing βarr2 in the nucleus accumbens of *Dbh -/-* mice reversed the cocaine hypersensitivity (Gaval-Cruz *et al.*, 2014), demonstrating the complex effects that βarr2 can have in cocaine-induced locomotion. These results are difficult to reconcile with the data obtained from βarr2-KO mice, which show minimal or no blunting of cocaine-induced locomotion and significantly reduced amphetamine-induced locomotion, suggesting that overexpression of βarr2 would potentiate, not reduce, cocaine-induced locomotion. One possible explanation is that *Dbh -/-* mice have unique neural adaptations due to their chronic, lifelong loss of norepinephrine. An alternative explanation is that βarr2 in the nucleus accumbens functions in a bimodal way, such that abnormally high or low levels of βarr2 could blunt psychostimulant responses. Finally, whether *Dbh -/-* mice have reduced βarr2 in D1-, D2-, or both D1- and D2-containing MSNs is currently unknown, and the ratio of βarr2 in these two cell populations could potentially influence cocaine-induced locomotion.

Alcohol

βarr2-KO mice have been used to test the role of βarr2 in alcohol reinforcement. Whereas one study found that mice completely lacking βarr2 consumed significantly less alcohol at low doses and had a reduced preference for alcohol in a two-bottle choice procedure compared to controls (Bjork *et al.*, 2008), another lab observed that βarr2-KO mice consumed more alcohol, especially at higher doses (Li *et al.*, 2013). However, both studies reported that βarr2-KO mice had enhanced CPP for low doses of alcohol (Bjork *et al.*, 2013; Li *et al.*, 2013), possibly indicating that the lack of βarr2 conferred hypersensitivity to the rewarding properties of alcohol.

βarr2-KO mice also had attenuated alcohol-induced locomotor responses compared to wild-type animals (Bjork *et al.*, 2008; Li *et al.*, 2013).

βarr2-KO mice regained their righting reflex slightly faster than controls following a large dose of alcohol (Li *et al.*, 2013) but had normal motor performance on a rotarod when under the influence of alcohol (Bjork *et al.*, 2008). In addition, while βarr2-KO mice had slightly reduced blood alcohol levels following a single alcohol injection, alcohol clearance rates were similar to wild-type mice (Bjork *et al.*, 2008; Li *et al.*, 2013). Combined, these experiments indicate that the differences in behavioral response of βarr2-KO mice to alcohol probably cannot be attributed to differences in the sedative properties of ethanol or its pharmacokinetics.

A few studies have examined genetic differences in βarr2 in relation to alcohol. Two lines of rats, "alcohol-preferring" and "alcohol-avoiding," have been bred based on their voluntary alcohol intake. Alcohol-preferring rats had elevated levels of βarr2 mRNA in the nucleus accumbens, dorsal striatum, and hippocampus, as well as higher βarr2 protein in the hippocampus compared to alcohol-avoiding rats (Bjork *et al.*, 2008). Interestingly, Bjork et al. uncovered a novel haplotype variant of the βarr2 gene that completely segregated between the two lines of rats and was highly correlated with ethanol consumption, and bioinformatic analysis revealed an expression quantitative trait locus for βarr2 in the brain regions that had elevated βarr2 mRNA (Bjork *et al.*, 2008). This is consistent with the observation that βarr2-KO mice voluntarily consumed less alcohol (Bjork *et al.*, 2008). Higher levels of βarr2 therefore predict greater ethanol consumption, whereas eliminating βarr2 reduces ethanol intake. Although genetic differences in βarr2 correspond to ethanol intake in rodents, no association between different polymorphisms of βarr2 and alcohol dependence has been observed in humans (Oneda *et al.*, 2010).

The reinforcing effects of alcohol are thought to be mediated by dopamine release in striatal regions as well as endogenous opioid systems. Consistent with this theory, βarr2-KO mice had enhanced alcohol-evoked dopamine release in the nucleus accumbens shell (Bjork *et al.*, 2013), which may contribute to the alcohol hypersensitivity. However, βarr2-KO mice lacked the alcohol-induced elevations in *c-fos* in the nucleus accumbens shell seen in control animals (Bjork *et al.*, 2008). While μ-opioid receptor binding and function did not differ between βarr2-KO and wild-type mice in drug-naïve conditions (likely due to low levels of receptor activation), alcohol induced greater μ-opioid receptor agonist stimulation in the dorsal striatum and amygdala of βarr2-KO mice (Bjork *et al.*, 2013). These differences in μ-opioid receptor function may contribute to the altered behavioral responses to alcohol in βarr2-KO mice. The impaired desensitization and internalization of μ-opioid receptors in the absence of βarr2 may cause heightened μ-opioid receptor signaling and greater sensitivity to alcohol, as evidenced by enhanced CPP and reduced ethanol intake.

In addition to μ-opioid receptors, δ-opioid receptors can also interact with βarr2 and modulate alcohol intake. δ-opioid receptor agonists that strongly recruit βarr2 have been shown to increase ethanol intake in wild-type mice, whereas agonists with poor βarr2 recruitment dose-dependently decreased ethanol consumption (Chiang *et al.*, 2016). Furthermore, an agonist with low βarr2 recruitment decreased ethanol intake in βarr2-KO mice, whereas an agonist that robustly recruits βarr2 neither decreased ethanol intake nor blocked the development of a CPP for alcohol in βarr2-KO mice (Chiang *et al.*, 2016). These results indicated that the effect of low βarr2-recruiting δ-opioid receptor agonists to decrease alcohol consumption occurs through βarr2-independent mechanisms, whereas high βArr2-recruiting δ-opioid agonists require βarr2 to enhance alcohol intake. Together, this series of experiments demonstrates that the ability of Δ-

opioid receptor agonists to recruit βarr2 has significant effects on alcohol intake. Examining βarr2 recruitment of different δ-opioid agonists is critical because these compounds are being considered as treatments for alcoholism and depression, and giving a high βarr2-recruiting δ-opioid agonist could potentially exacerbate alcoholism rather than attenuate it.

Nicotine

Very few published studies have examined the influence of βarr2 on nicotine responses. In adolescent mice, nicotine caused hypolocomotion in both wild-type and βarr2-KO animals, and βarr2-KO mice appeared to be more sensitive to this effect (Correll *et al.*, 2009). However, as previously noted, genotype differences in baseline locomotor activity make these results difficult to interpret. βarr2-KO mice also exhibited impaired nicotine-induced locomotor sensitization; whereas repeated nicotine administration significantly increased locomotion in adolescent control mice, adolescent nicotine-induced locomotor activity was stable over time in βarr2-KO mice (Correll *et al.*, 2009). Future experiments are needed to determine whether these βarr2-associated changes in locomotor activity are also reflected in nicotine reward.

As with opioid-dependent individuals, certain polymorphisms of βarr2 have been associated with nicotine users. In European Americans but not African Americans the rs4790694 SNP of βarr2 significantly correlated with the Heaviness of Smoking Index and the Fagerström Test for Nicotine Dependence (Sun *et al.*, 2008). This finding demonstrated that particular polymorphisms of βarr2 can confer risk of nicotine dependence in certain human populations. Further research is needed to determine the biological effects of this polymorphism on βarr2 function in neurons. Note that this is not one of the same SNPs that predicted drug relapse while on methadone maintenance therapy (Oneda *et al.*, 2011).

Hallucinogens

Hallucinogenic effects are mediated by the serotonin (5-HT) system, particularly the 5-HT_{2A} receptor. Studying hallucinations in rodents is difficult due to the cognitive nature of hallucinations; however, rodents treated with hallucinogens exhibit a head twitch that can be quantified, and βarr2 is involved in this behavioral response. Unlike control mice, βarr2-KO mice did not exhibit a head twitch response to moderate doses of the 5-HT precursor 5-HTP; however, βarr2-KOs displayed a normal head twitch to the 5-HT_{2A} receptor agonist DOI, and had more head twitches than wild-type mice at high doses of 5-HTP (Schmid *et al.*, 2008; Schmid & Bohn, 2010). Additionally, 5-HT, but not N-methyltraptamines, engaged a βarr2/phosphoinositide 3-kinase/Src/Akt cascade in cortical neurons (Schmid & Bohn, 2010). Together, these experiments indicate that both N-methyltraptamines and 5-HT induce head twitch via the 5-HT_{2A} receptor, but do so through different mechanisms. βarr2 promotes the 5-HT-induced response, but attenuates the N-methyltryptamine-induced response (Schmid & Bohn, 2010). These experiments highlight the importance of functional selectivity of different agonists at the same receptor.

Similar to other hallucinogens, the effects of lysergic acid diethylamide (LSD) are primarily mediated by the serotonin 5-HT_{2A} receptor (De Gregorio *et al.*, 2016), although LSD binds to most serotonin receptors and some other GPCRs (Kroeze *et al.*, 2015). A few studies have used the 5-HT_{2B} receptor, which is very similar to the 5-HT_{2A} receptor, to study the molecular effects of LSD. Compared to 5-HT and other agonists, LSD is strongly biased towards βarr2 signaling over G protein signaling at the 5-HT_{2B} receptor (Wacker *et al.*, 2013), which may contribute to its hallucinogenic effects (Chen & Tesmer, 2017). Recently, the crystal structure of LSD bound to the 5-HT_{2B} receptor was described, and 5-HT_{2A} receptor models show similar

binding properties. Interestingly, a portion of an extracellular loop of the receptor (EL2) formed a "lid" over LSD, which prevented LSD from dissociating from the receptor and may be responsible for LSD's long duration of action. Indeed, mutating part of the lid ($L209^{EL2}$) resulted in a 10-fold reduction in the duration of time that LSD was bound to the receptor. Additionally, this mutation significantly decreased LSD's recruitment of βarr2 without altering Gq-mediated calcium flux (Wacker *et al.*, 2017). These results indicate that LSD recruits βarr2, and future experiments should examine the behavioral consequences of βarr2 in the hallucinogenic effects of LSD.

A few experiments have examined βarr2's role in phencyclidine (PCP) effects in the context of schizophrenia, because PCP can induce psychotic-like effects (Urs *et al.*, 2017). PCP robustly increased locomotor activity in wild-type and βarr2-KO mice (Allen *et al.*, 2011). βarr2-biased D2 receptor agonists, such as UNC9994, have been developed and have antipsychotic-like properties. UNC9994 attenuated PCP-induced locomotion in wild-type mice but had no effect in βarr2-KO mice, demonstrating functional selectivity of the ligand (Allen *et al.*, 2011). Injecting UNC9994A into mouse prefrontal cortex inhibited PCP-induced locomotion, and eliminating βarr2 in D2-containing neurons prevented this effect. βarr2 in non-striatal D2 neurons, such as the cortex, likely mediates PCP-induced locomotion because eliminating βarr2 only in D2-containing striatal neurons did not alter the ability of UNC9994A to inhibit PCP-induced locomotion, but virally removing βarr2 in cortical D2 neurons abolished the drug's effect (Urs *et al.*, 2016). In addition to functional selectivity, UNC9994A caused different effects in D2 receptor-containing neurons in the striatum versus prefrontal cortex. Whereas UNC9994A did not affect the excitability of striatal MSNs, UNC9994A increased fast-spiking interneuron excitability in the prefrontal cortex, and this effect was mediated by elevated GRK2 expression

(Urs *et al.*, 2016). The finding that a biased βarr2 ligand at D2 receptors functions as an agonist in prefrontal cortex but not in the striatum demonstrates the importance of brain region-specific properties of βarr2.

Summary

Most rodent experiments examining the contribution of βarr2 to the effects of addictive drugs have used conventional βarr2-KOs. Completely eliminating βarr2 causes changes in the behavioral responses to most classes of drugs of abuse, but whether it blunts, potentiates, or has no effect varies by assay (drug-induced locomotion versus CPP) and drug (Table 1). In the instances where complete loss of βarr2 has no significant reported effects, such as with cocaine, there are a few potential explanations: βarr2 could be uninvolved, βarr1 could be compensating for the lack of βarr2, and/or the assays used lacked sufficient sensitivity to detect a βarr2-KO phenotype. Whole-body deletion of βarr2 typically blunts opioid, psychostimulant, nicotine, and ethanol-induced locomotion; however, loss of βarr2 can potentiate opioid and alcohol reward. Together, these findings indicate that βarr2 is differentially involved in the rewarding versus locomotor-activating effects of drugs and support the notion that locomotor responses to drugs do not always reliably predict their rewarding properties.

The most thorough investigations of βarr2's involvement in drug effects thus far have been conducted with morphine and amphetamine. βarr2 signaling, particularly in D2-containing MSNs, mediates amphetamine-induced locomotion (Peterson *et al.*, 2015; Urs *et al.*, 2016); however, whether this mechanism also mediates amphetamine reward is currently unknown. By contrast, βarr2 in D1-containing neurons is proposed to mediate morphine-induced locomotion but not reward (Urs *et al.*, 2011; Urs & Caron, 2014). While a simplistic model whereby βarr2

mediates all psychomotor drug effects would be convenient, the data indicate that βarr2 is likely modulating different drug effects in different brain regions through interactions with various GPCRs and intracellular molecules. Much work remains to be done to elucidate how βArr2 modulates the behavioral effects of addictive drugs.

Technical Advances and Limitations

βarr2-KO mice have been a valuable tool for testing the global effects of βarr2. As discussed earlier, however, genetic deletion carries the caveat that adaptations during development may mask, enhance, or otherwise alter βarr2-associated phenotypes. Additionally, βarr2-KO mice obscure regional, cell type-, and receptor-specific effects. Conditional knockouts whereby βarr2 is eliminated in D1- or D2-contianing cells by crossing floxed βarr2 mice with D1Cre or D2Cre mice (Urs *et al.*, 2016) provide enhanced cell type specificity but still lack regional specificity because D1 and D2 receptors are expressed in multiple brain regions. The floxed βarr2/A2aCre mice, which lack βarr2 in D2-containing MSNs (Urs *et al.*, 2016), provide enhanced regional and cell type specificity but do not differentiate between subregions of the striatum, which can have different roles in addictive-like behaviors (Everitt & Robbins, 2013). Depending on the promoter, viral elimination or overexpression of βarr2 can grant further regional and cell type specificity. Experiments using these techniques have focused primarily on knocking out βarr2 (Urs *et al.*, 2016), but using viral vectors to overexpress βarr2 is also possible (Gaval-Cruz *et al.*, 2014).

Viral vectors that express GPCRs with biased βarr2 signaling (Peterson *et al.*, 2015) are a useful tool for dissociating the effects of receptor activation and engagement of multiple signaling pathways with the specific effects of βarr2 in the cells containing that receptor. D2

receptors that preferentially activate βarr2 signaling or traditional Gi/o signaling can be used to dissect the importance of one pathway over the other (Peterson *et al.*, 2015), and are useful for testing the *in vivo* effects of D2 receptor-mediated activation of each pathway. Developing mutated D1, μ-opioid, and cannabinoid receptors that preferentially induce G protein or βarr2 signaling would also be beneficial for future experiments examining the role of βarr2 in drug effects. The limitation of biased signaling experiments is that they currently require viral infusions that typically result in overexpression of the GPCRs, which may or may not reflect natural βarr2 signaling and function.

Although direct agonists/antagonists of βarr2 are not yet available, biased ligands that are receptor agonists preferentially engaging βarr2 are useful tools for eliciting βarr2 signaling versus canonical G protein signaling. For example, there are D2 receptor ligands that function as partial agonists engaging βarr2 but not G protein signaling (Allen *et al.*, 2011) and κ-opioid receptor agonists that harness G-protein but not βarr signaling (Rives *et al.*, 2012). Additional drugs that specifically and acutely target βArr2 would be beneficial to more thoroughly investigate the role of this protein in modulating behavioral responses to drugs of abuse.

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), which use mutated receptors with selective ligands to activate or inactivate particular cells or signaling pathways, have become a popular tool in neuroscience research recently (Roth, 2016; Smith *et al.*, 2016). A DREADD has been invented that activates βarrs but not G proteins (Nakajima *et al.*, 2015), which could be very useful for preferentially activating βarrs without causing signaling from endogenous receptors. To our knowledge, this βarr DREADD has not yet been employed to investigate βarr2 contributions to behavior or in relation to drugs of abuse.

Another limitation in studying βarr2 is the difficulty in visualizing the protein. Immunohistochemistry and western blot experiments have suffered from a lack of commercially available, sensitive, specific βarr2 antibodies. Some investigator-produced antibodies have yielded better results (Urs *et al.*, 2016), but these resources are limited. Recently, βarr2 biosensors have been developed to detect in vitro real-time conformational changes in βarr2 and the "megaplexes" that can form combining internalized GPCR, βarr2, and G protein (Nuber *et al.*, 2016). These sensors could be useful in visualizing the precise conformational changes and intracellular movements of βarr2 in response to drugs of abuse.

Surprisingly, only a few experiments have used electrophysiology to examine how βarr2 manipulations affect the excitability of neurons within reward circuits. Enkephalin hyperpolarized locus coeruleus neurons to the same degree in βarr2-KO and control neurons (Arttamangkul *et al.*, 2008). Interestingly, the βarr2-biased D2 ligand UNC9994A had little effect on striatal MSNs but increased the excitability of fast-spiking interneurons in the prefrontal cortex. This increased activity in the cortex was attenuated in βarr2-KOs, indicating that βarr2 mediates the effects of UNC9994A on cell excitability (Urs *et al.*, 2016). Additionally, whereas the D2 agonist quinpirole normally decreases the excitability of MSNs, it increased the excitability of MSNs in *Dbh -/-* mice that have decreased βarr2 in the nucleus accumbens (Gaval-Cruz *et al.*, 2014). These experiments hint at the complex effects that βarr2 can have on neural activity in different brain regions. Future experiments should investigate psychostimulant- and opioid-induced changes in the excitability of D1- and D2-containing MSNs and prefrontal cortical neurons following βarr2 manipulations. Additionally, βarr2's role in synaptic plasticity following drug use should be examined.

Because the defining symptoms of drug abuse are behavioral, biological targets for treating drug abuse must be able to change either the reinforcing properties of drugs or the motivation to take drugs. Most experiments examining the behavioral effects of βarr2 manipulations have used simple behavioral procedures, such as drug-induced locomotion, locomotor sensitization, or conditioned place preference. These behavioral tasks are useful screens for measuring drug responses because they are reliable, technically simple, inexpensive, and quantitative; however, on their own, they are inadequate assays for addiction. Future studies should use operant drug self-administration procedures that can answer more sophisticated behavioral questions probing voluntary intake, escalation of intake, drug consumption despite adverse consequences, and relapse-like behavior.

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Footnotes

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Table 1. The Behavioral Effects of Drugs of Abuse in Mice Completely Lacking βarr2.

Drug	**Drug-Induced Locomotion**	**Locomotor Sensitization**	**Conditioned Place Preference**
Cocaine	=	?	=
Amphetamine	↓	?	?
Morphine	↓	=	↑
Alcohol	↓	?	↑
Nicotine	=↓	↓	?
Cannabinoids	?	?	?

= similar to controls, ↓ blunted compared to controls, ↑ enhanced compared to controls, ? currently unknown.